Effect of Photoperiod and Acute Stress on Facilitative Glucose Transporter GLUT3 in Siberian Hamsters (*Phodopus sungorus*)

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Metabolic activity in mammalian brains is dependent upon glucose utilization. Transport of glucose to the neurons requires mediation by a family of known as facilitative glucose transporter proteins (GLUTs). The GLUT3 isoform is solely responsible for facilitating glucose transport in mammalian brains. Studies show that age-related brain dysfunction and disease, as well as reductions in learning and memory correlate with decreased GLUT3 expression. Winter is a particularly difficult time to reproduce and survive because of the energy bottleneck caused by the coincidence of low ambient temperatures with reduced food availability. Individuals cope with this energetic bottleneck by engaging tradeoffs among expensive physiological processes through development of specific adaptations including adjustments in metabolic rate, body mass, reproduction, and immune function. Non-tropical animals determine time-of-year by monitoring photoperiod (day length) in order to evoke the appropriate suite of adaptations. However, such metabolic trade-offs can be challenged by stressors. To test the combined effects of acute stress and photoperiod on GLUT3 expression, male adult *Phodopus sungorus* were housed in either long or short photoperiods for 14 weeks. After 14 weeks, half of the animals from each photoperiod were subjected to restraint stress immediately prior to brain collection. Brain tissue from each animal was excised and used in GLUT3 gene expression analysis. The findings of this study demonstrate that photoperiod and acute stress do not impact the facilitative glucose transporter GLUT3 at the RNA expression level. Further research needs to be conducted in order to determine the precise mechanism which regulates the expression of the GLUT3 transporter in neurons of the hippocampus.

Introduction

The primary energy source for mammalian brains is the simple carbohydrate glucose. The polarity of the glucose molecule necessitates the need for a protein to assist the transport of glucose across the non-polar neuronal cell membrane. The transport of glucose into the cell is mediated by a family of integral membrane proteins known as facilitative glucose transporter proteins, commonly referred to as GLUTs. This set of facilitative glucose transporters is commonly grouped into a gene family known as SLC2. The SLC2 family of GLUT transporters is further broken down into three classes. Class I contains the more well studied glucose transports GLUT1, GLUT2, GLUT3, and GLUT4. Class II contains GLUT5, GLUT7, GLUT9, and GLUT11. Class III contains the remaining transporters – GLUT6, GLUT8, GLUT10, GLUT12, and HMIT (GLUT13) (Manolescu, Witkowska, Kinnaird, Cessford, Cheeseman, 2007). The GLUT1 isoform is

primarily responsible for transport of glucose from the periphery across the blood-brain barrier into the vasculature of the central nervous system. The GLUT4 isoform is an insulin sensitive glucose transport protein found in both the cerebellum and hypothalamus, while the GLUT5 isoform is found predominantly in microglia.

Of particular interest to neuroscientists is the GLUT3 isoform (SLC2A3) because it is the primary glucose transporter in neurons of mammalian brains, and is very highly concentrated in areas of high synaptic density (Fattoretti et al., 2001). Because of its fundamental role in neuronal glucose metabolism, GLUT3 has been implicated as a possible major factor in the development of neurodysfunction and disease. A number of previous studies have explored the effects of different stimuli on the expression of GLUT3 transporter, as well as the implications of decreased GLUT3 transporter presence in the mammalian brain. Previous studies report that a decrease in GLUT3 concentration in neurons of the medial temporal lobe may be responsible for age-related metabolic deficits seen in this area, leading to deficits in learning and memory. Neurodegenerative diseases such as Alzheimer's disease show this characteristic decrease in cerebral metabolic activity (Simpson et al., 2008). Reagen and colleagues (1999) showed that 6 h of restraint stress daily for 7 days was not effective in decreasing the expression of GLUT3 mRNA or GLUT3 protein in the hippocampus of non-diabetic rats. Diabetes alone is capable of increasing the expression of both GLUT3 mRNA and GLUT3 protein in the hippocampus and this increase in expression was further exacerbated when coupled with 7 days of restraint stress (Reagen et. al., 1999), suggesting the increase may be a compensatory mechanism to increase glucose utilization in diabetic mammals and may also have a stress-response component.

Stressful experiences have a pronounced effect on physiological processes in mammals. These effects are transmitted via endocrine and nervous activation of a number of signaling pathways which provide the animal with salient information regarding the stressful incident, and direct the animal's response. Adaptations have evolved that allow animals to effectively cope with the energetic demands presented by balancing the activity of a number of physiologically expensive processes, and most effectively utilizing their available energy stores. The major hormonal components of the energy conservation pathways of the stress response are epinephrine (adrenaline) and gluccocorticoids. Epinephrine, a catecholamine produced and secreted by the adrenal glands, acts via the "fight-or-flight" response pathway of the sympathetic nervous system by increasing the concentration of blood glucose and fatty acids, and preventing glucose storage in tissue cells,   providing

animals with increased substrates for energy production. Epinephrine also increases the levels of adrenocorticotropic hormone (ACTH) secreted by the anterior pituitary gland. Gluccocorticoids are a class of steroid hormones synthesized and released by the adrenal cortex in response to the release of ACTH from the anterior pituitary via signaling of the Hypothalamic-pituitary-adrenal (HPA) axis. In some small mammals, such as Siberian hamsters (*Phodopus sungorus*), the primary gluccocorticoid released in response to stress is cortisol (Reuben, Wynne-Edwards, 1999). Cortisol is responsible for increasing rates of glucose metabolism by promoting gluconeogenesis and preventing the uptake of glucose into muscle and adipose tissue.

Winter is a particularly difficult time to reproduce and survive because of the energy bottleneck caused by the coincidence of low ambient temperatures with reduced food availability. Animals such as the Siberian hamsters cope with this seasonal energetic bottleneck by engaging in tradeoffs among expensive physiological processes through development of specific adaptations including adjustments in metabolic rate, body mass, reproduction, and immune function. Non-tropical animals determine time-of-year by monitoring photoperiod (day length) in order to evoke the appropriate suite of adaptations to enable the greatest probability of survival. One of the energetically taxing processes often reduced in response to decreased availability of resources is reproductive function. Reduction in winter breeding is a hallmark of the suite of adaptations normally evoked by non-tropical animals in an effort to preserve their increasingly scarce energetic resources. A number of mammalian cell lines are able to adaptively respond to situations necessitating large amounts of energy by increasing their glucose utilization levels to meet their current energetic demands (Khayat, McCall, Klip, 1998). We hypothesized that cellular GLUT3 expression may be a critical factor in mediating the ability of mammals to respond to the metabolic demands of their environment. However, such metabolic trade-offs can be challenged by stressors.

During winter months animals will normally display increased basal levels of gluccocorticoids as well as produce more pronounced stress responses when compared to summer months. However, it has been observed that because of adaptive processes employed in anticipation of decreased energy availability animals will not show exaggerated stress responses during winter months. Seasonal differences in gluccocorticoid concentrations *in situ* have been in observed in *P. sungorus* housed in laboratory settings with variable photoperiods, with animals housed in short photoperiods displaying increased basal gluccocorticoid concentration (Nelson, Martin 2007). Gluccocorticoids are effective in decreasing the rate of glucose uptake into hippocampal

cell cultures (Homer, Packan, Sapolsky, 1990). Although the effects of glucocorticoids on physiological processes involving glucose metabolism have been studied, it is yet to be determined whether increased glucocorticoid concentration is effective in altering the expression of the GLUT3 transporter in the brain tissue.

The primary goal of the study was to determine what correlation, if any, exists between photoperiod and stress levels in determining the expression of the GLUT3 transporter in the mammalian brain. Determining whether a correlation exists among acute stress-induced release of cortisol, photoperiod, and expression of the GLUT3 transporter will allow for further understanding of how the expression of the GLUT3 transporter is regulated *in situ*. Understanding the mechanisms underlying GLUT3 expression may lead to more effective counter-degenerative steps being taken to prevent hippocampal cell loss, preventing further development of neurodysfunction and disease corresponding to a decrease in cerebral metabolic activity.

Materials and Methods

Animals

Forty adult male Siberian hamsters (*Phodopus sungorus*) from the breeding colony of Dr. Randy Nelson were used in this study. Animals were individually assigned to one of four experimental conditions which outlined the stress and photoperiod paradigm to which they would be subjected. Twenty hamsters were housed in long day (LD) conditions with a 16:8 light/dark cycle (lights on 2200 h Eastern Standard Time, EST), and 20 hamsters were housed in short day (SD) conditions with a reverse 8:16 light/dark cycle (lights on at 0600 h EST). Within each photoperiod group was a stress group (S, n=20) and a non-stress group (NS, n=20). Animals were housed individually in polypropylene cages (27.8 x 7.5 x 13 cm) in colony rooms with constant temperature and humidity of 21 ± 4C and 50 ± 10%, respectively, and had *ad libitium* access to food (Harlan Teklad 8640 rodent diet, Indianapolis, IN) and filtered tap water. All hamsters remained in their respective photoperiod conditions for 14 wk prior to being subjected to their respective experimental stress conditions. All hamsters were weighed weekly to monitor changes in body mass. Food intake was also monitored weekly to determine changes in food consumption in response to photoperiod. All procedures were approved by the Ohio State University Institutional Animal Care and Use Committee and are in compliance with guidelines established by the National Institutes of Health published in *Guide for the Care and Use of Laboratory Animals* (1996).

Acute Stress Treatment

Animals to be stressed (LD S, n=10, SD S, n=10) were removed from their cages and placed into clear, well-ventilated polypropylene restraint tubes in a quiet room at 0700 h EST and remained restrained until 11 AM EST. After completion of the 4 h of restraint stress, each animal was removed and immediately sacrificed.

Tissue Collection

At the conclusion of week 14 all hamsters were deeply anesthetized with 5% isoflurane (Butler Animal Health, Dublin, OH) via inhalation. A final weight was then taken on each animal. Approximately 0.25 ml of blood was drawn from each animal using a retro-orbital sinus bleed. Immediately after receiving the retro-orbital sinus bleed each hamster received a lethal dose (60mg/60kg) of sodium pentobarbital (Henry Schein, Melville NY) via intraperitoneal injection (vol = 0.2ml). Reproductive tissues (testes, epididymides, and seminal vesicles) were removed and weighed to verify reproductive responses to photoperiod. Brains of all hamsters were removed and sagittaly sectioned down the midline. Right hemisphere sections were immediately flash frozen on dry ice and stored at -80° C until processing. Left hemisphere sections were placed in RNALater solution (Qiagen, Valencia, California) and stored at 4° C until RNA processing.

Radioimmunoassay

Blood obtained from each animal via retro-orbital sinus bleed immediately before death was centrifuged at 6,000 rpm, for 30 min at 4° C in order to obtain serum samples for analysis. Serum samples were assayed for cortisol using radioimmunoassay (RIA). The RIA was conducted according to manufacturer's suggested protocol using a 125I double antibody kit (Diagnostic Products Corporation, Los Angeles, CA). Each sample was run in duplicate and the mean cortisol concentration values of each animal's serum sample were recorded. The assay had an upper limit of 6.3591, a lower limit of 196.64, and a coefficient of variation <10%.

RNA Processing

The hippocampus was dissected from each left hemisphere section and individually homogenized (Power Gen 1000, Fischer Scientific, Pittsburgh, PA) to obtain total RNA using an RNeasy Mini Kit according to the manufacturer's protocol (Qiagen). RNA samples were suspended in 30 µL of RNAse-free water and RNA concentration was determined
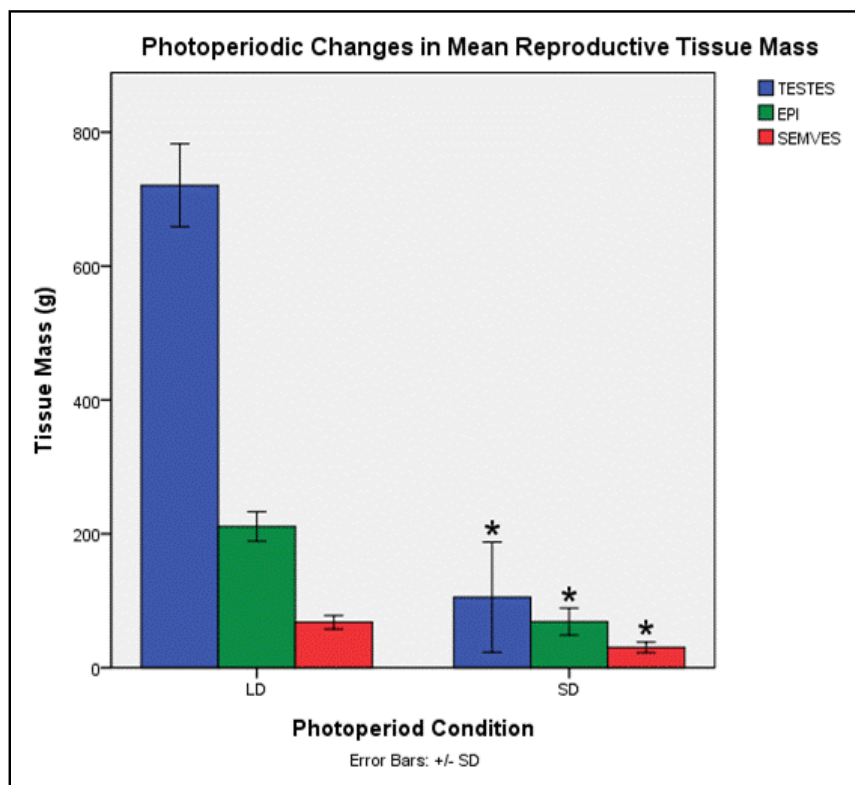
Fig 1 Photoperiod response was confirmed by comparing mean reproductive tissue mass between groups. Short days significantly lowered paired testes, paired epididymal, and paired seminal vesicle mass (all p < 0.05) compared to long days.
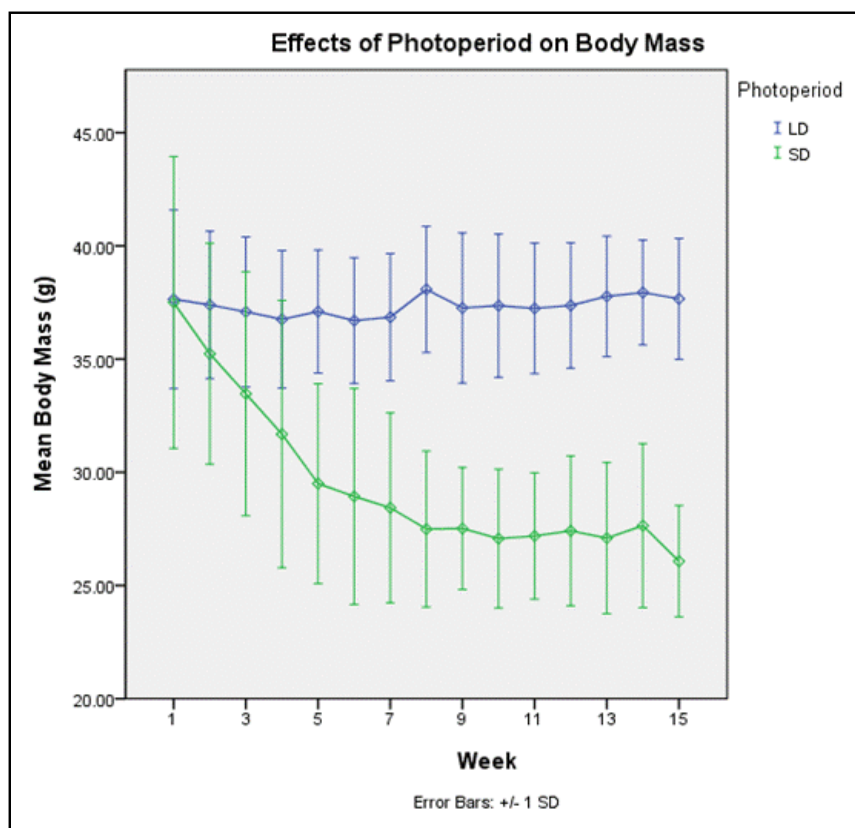
Fig 2 A repeated measures ANOVA was used to test the effects on body mass of LD and SD photoperiod conditions. There was an effect of photoperiod on body mass (p < 0.05).

using a spectrophotometer (NanoDrop 1000). RNA samples were stored at 80° C until further analysis. cDNA was created via reverse transcription of 2 µg of total RNA from each sample with MMLV Reverse Transcriptase enzyme (Invitrogen, Carlsbad,CA, USA) according to the manufacturer's suggested protocol.

Gene Sequencing

In order to design probes and primers to be used in quantitative real-time PCR (qRT-PCR) the SLC2A3 gene was first sequenced. In order to sequence the gene of interest, 1 µL of brain cDNA was used in conjunction with Invitrogen PCR Super Mix (Invitrogen) to run semi-quantitative PCR for 35 cycles in a thermocycler (Bio-Rad, Hercules, CA) using the following cycling conditions: 94° C for 2 min, followed by 35 cycles of 94° C for 30 min, 56° C for 30 min, 72° C for 30 min, and ending with 72° C for 7 min. Conserved code regions among multiple species with the known GLUT3 gene sequence (Gene Bank) were used to design degenerate primers using Primer Express software (Applied Biosystems, Carlsbad, CA). Amplification of the PCR gene product was visualized on 2% TAE-agarose gel containing GelRed Nucleic Acid Stain (Phenix Research, Candler, NC) using a CCD camera. In order to verify the amplification of the GLUT3 gene, PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen) and sequenced at the Plant-Microbe Genomics Facility at The Ohio State University. The resulting sequences which were >90% homologous to the *Mus* gene of interest were assumed to be the correct *P. sungorus* gene of interest.

qRT-PCR

After confirming the sequence of the GLUT3 gene, primers and a probe were designed using Primer Express (Applied Biosystems). Sequences of the primers and probe used are listed below. The probe was labeled with 6-FAM (fluorescent dye) at the 5' end and MGB (non-fluorescent quencher) at the 3' end.

SLC2A3 Forward Primer:
    5'-GTGGCATGATGGGCTCTTTT-3"
SLC2A3 Reverse Primer:
    5'-GAGTTGCGTCTGCCAAAGC-3"
SLC2A3 Probe:
    5'- CTGTTGGACTCTTTG-3'

A TaqMan 18S Ribosomal RNA primer and probe set labeled with VIC (Applied Biosystems) was used to as the control gene for determining relative quantification of the target gene. An ABI 7000 Sequencing Detection System and Taqman Universal PCR Master Mix were used in performing the amplification process. The universal two-step RT-PCR cycling conditions used were 50° C for 2 min, 95° C for 10 min, followed by 40 cycles of 95° C for 15 s and 60° C for 1 min. Each sample was run pseudo-randomly in duplicate on one plate. Relative gene expression was determined by comparison to a relative standard curve created using pooled *P. sungorus* hypothalamic cDNA of different concentrations (1:10. 1:100. 1:1000, 1:10000) which was normalized using 18S rRNA gene expression.

Results

Photoperiod Response

Photoperiod response was confirmed by comparing mean reproductive tissue mass between groups. Short days significantly lowered paired testes, paired epididymal, and paired seminal vesicle mass (all $p < 0.05$) compared to long days (See Figure 1). Significant differences in pelage were also observed between groups ($p < 0.05$) (See Figure 2). A repeated measures ANOVA was used to test the effects on body mass of LD and SD photoperiod conditions. There was an effect of photoperiod on body mass, LD mean 37.85 g ± 4.41. SD mean 30.62 ± 3.78; $F_{(1, 12)} = 8.00$, $p < 0.05$ (See Figure 3).

Acute Stress Response

A one-way ANOVA was used to test for differences in blood cortisol levels among the 4 groups. Cortisol concentrations differed significantly between the stress groups and non-stress groups for both photoperiods, $F_{(3, 36)} = 5.618$, $p = 0.003$. Tukey post-hoc comparisons between the groups showed a significant increase in blood cortisol between the LD S group (M=66.07, 95% CI, [35.97, 96.10]) and the LD NS group (M=31.63, 95% CI, [22.83, 40.43]), p=0.027 and the SD NS group (M=32.97, 95% CI, [22.46, 43.12]), p=0.034. Tukey post-hoc comparisons also show a significant increase in blood cortisol between the SD

S group (M=65.84, 95% CI, [48.69, 82.99]) and both the SD NS and LD NS groups, p= 0.028, p=0.035, respectively (* - indiciates differences from NS group of same photoperiod). No significant difference was observed between the LD S and SD S groups or between the LD NS and SD NS groups at p < 0.05 (See Figure 4).
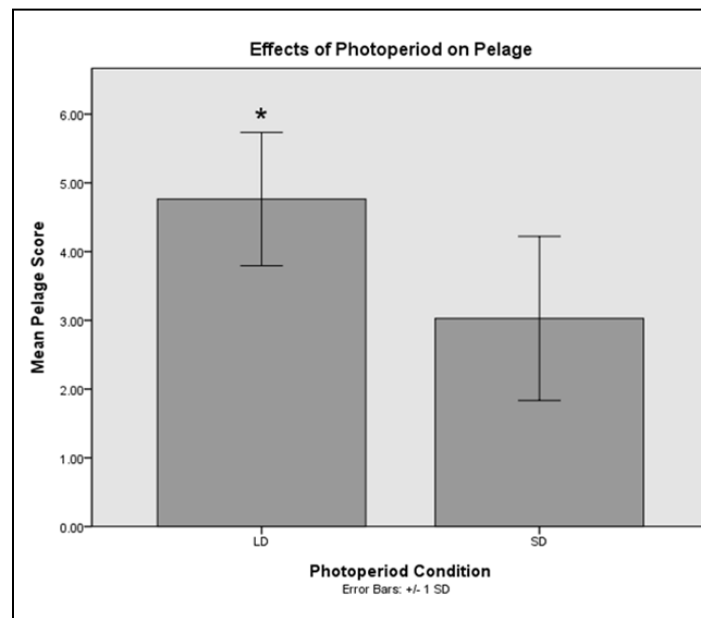

Fig 3 Significant differences in pelage were observed between groups (p < 0.05).

Gene Expression Analysis

A one-way ANOVA was used to test for differences in expression of the GLUT3 gene (SLC2A3) mRNA among the 4 groups. No significant differences were observed between any groups, $F_{(3, 36)} = 0.628$, $p = 0.602$. An independent samples *t*-test showed no significant differences between LD (M = 0.96, SD = 0.088) and SD (M = 0.99, SD = 0.062) conditions, $t(38) = -1.41$, $p = 0.261$. An independent *t*-test also showed no significant difference between NS (M = 0.97, SD = 0.065) and S (M = 0.98, SD = 0.088) conditions, $t(38) = -0.347$, $p = 0.730$). The Levene test of homogeneity of variance was not significant p =0.479, indicating variance among the groups did not differ and therefore established the one-way ANOVA as appropriate analysis among groups. An independent samples *t*-test comparing the LD NS (M = 0.95, SD = 0.070) and SD NS group (M = 0.99, SD = 0.053) showed nonsignificant, but noteworthy, results $t(38) = -1.64$, $p = 0.124$ (See Figure 5).

Discussion

This experiment was designed to test what effects photoperiod and acute stress have on the expression of GLUT3 transporter mRNA in the hippocampus of adult
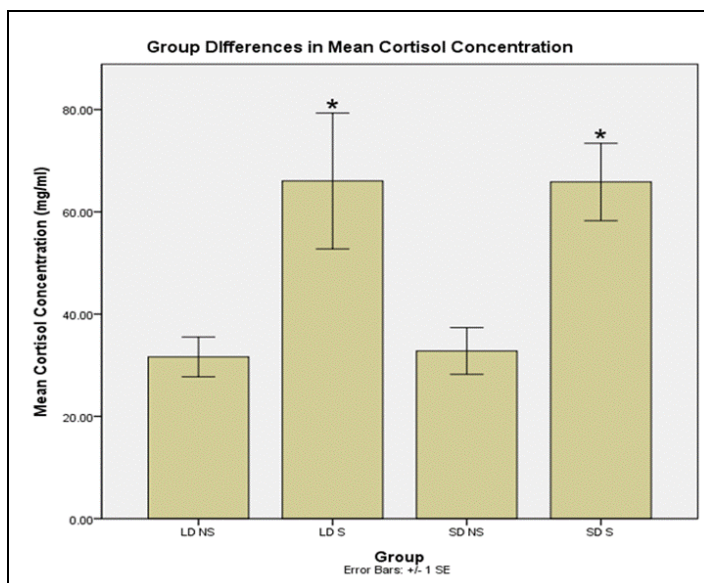
Fig 4 Cortisol concentrations differed significantly between the stress groups and non-stress groups for both photoperiods (p = 0.003).
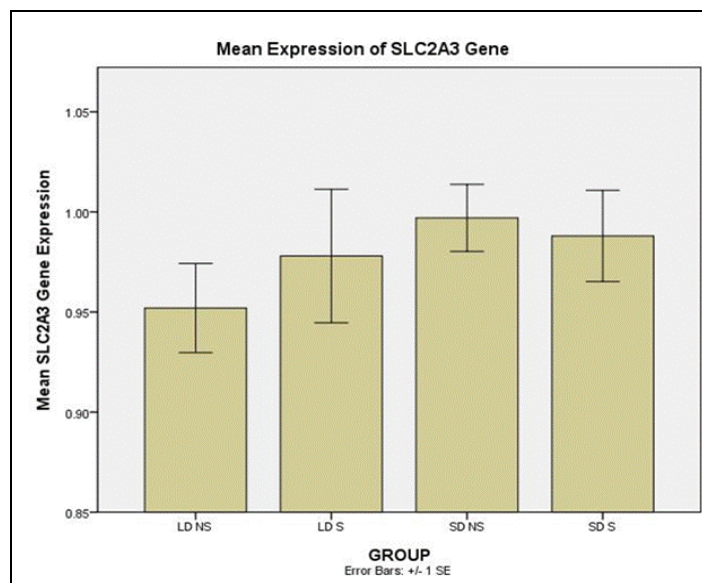
Fig 5 Neither photoperiod nor acute stress-induced cortisol concentration were shown have to significant effects on the expression of the GLUT3 (SLC2A3) RNA in the hippocampus.

mammals. It is believed that GLUT3 transporter may play a critical role in the ability of neurons to cope with their energetic demands by facilitating the transport of glucose into the cell for use in metabolic processes. This transport is believed to be affected by certain exogenous stimuli acting on physiological processes related to glucose availability and metabolism

Analysis of photoperiod specific differences in mean reproductive tissue mass and pelage score show that photoperiod was effective in eliciting significant reduction in reproductive state in animals housed in SD compared to those housed in LD. In response to photoperiod many animals will decrease their food-intake and body mass in anticipation of growing energy scarcity. *P. sungorus* voluntarily reduces food-intake which leads to a significant reduction in their body mass in response to changes in photoperiod conditions in anticipation (Knopper, Boiley, 2000; Dark, Zucker, Wade, 1983). A significant reduction in body mass was also observed in the results of this study, with SD animals showing a significant decrease in body mass as a result of environmental changes. These combined results confirm the positive response to animals housed in SD photoperiod led to a significant decrease their overall reproductive state, most likely in anticipation of available energy scarcity.

Cortisol is known to increase rates of glucose metabolism by promoting gluconeogenesis and preventing the uptake of glucose into muscle and adipose tissue. Differences in mean blood cortisol concentration between S and NS groups showed a significant increase in circulating blood cortisol in animals exposed to 4 h of acute restraint stress compared to NS controls. Data show a significant increase between photoperiod conditions, with LD S showing significant

increases in serum cortisol compared to LD NS and SD NS, as well as SD S showing significant increases in serum cortisol compared to both SD NS and LD NS. These data show that 4 h of acute restraint stress was successful in eliciting the expected endocrine stress response through increasing cortisol concentration which will in turn affect rates of glucose metabolism *in situ*.

Neither photoperiod nor acute stress-induced cortisol concentration were shown have to significant effects on the expression of the GLUT3 (SLC2A3) RNA in the hippocampus. Results of the study show no significant differences between any of the 4 experimental groups. The p-value for data comparing the effects of photoperiod alone do however show an interesting, yet statistically nonsignificant, trend of increased expression of the SLC2A3 gene in SD animals. Although significant differences in RNA expression were not observed in the hippocampus using this paradigm, it does not completely negate the idea that these factors can impact the expression of the GLUT3 transporter. The regulatory mechanism at work may act through other pathways such as inactivation of expressed transporter at the plasma membrane, or through sequestering membrane bound transporter to intracellular storage sites as is observed in other GLUT isoforms. A previous study examining the effects of insulin-induced changes in glucose transport showed that increases in insulin were sufficient to cause translocation of glucose transporters in human adipose cells (presumably GLUT4, although not explicitly stated in the text) from intracellular storage pools to the plasma membrane (Karnieli, Barzilai, Rafaeloff, Armoni, 1986). It has also been shown that dexamethasone (a synthetic gluccocorticoid class steroid hormone) was effective in decreasing the expression

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of plasma membrane glucose transporter in fibroblast cells, without decreasing the total amount of glucose transporter present in the cell (Horner, Munck, Lienhard, 1987). A cell specific regulatory mechanism of the neuron specific GLUT transporter differing from that of the glial cell regulatory mechanism, which occurs at the RNA expression level, has also been suggested previously (Walker, Donovan, VanNess, Fellows, Pessin, 1988).

In conclusion, the current findings demonstrate that photoperiod and acute stress do not impact the facilitative glucose transporter GLUT3 at the RNA expression level. Considering the results of this study and the other relevant studies conducted it is reasonable to suggest that regulation of the neuron specific GLUT3 transporter concentration may occur at the protein level rather than at the RNA level in response to elevated gluccorticoid levels *in situ*. The expression of GLUT3 transporter protein in the tissue samples collected was not able to be analyzed due to the time constraints which existed for the study. Further research needs to be conducted to determine whether the proposed membrane translocation mechanism does in fact exhibit regulatory control over GLUT3 transporter protein plasma membrane concentration, and if it does, what pathways this mechanism employs in sequestering and releasing GLUT3 membrane transporter from intracellular storage pools.

Acknowledgements

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